

AMENDMENTS TO THE SPECIFICATION

Applicants have amended the following paragraphs in the Specification:

Page 1, lines 6-11.

This application claims priority to Kamal et al., PCT/US03/18776, filed June 12, 2003, entitled CYTOTOXINS AND DIAGNOSTIC IMAGING AGENTS COMPRISING HSP90 LIGANDS, which claims priority to Kamal et al., PCT/US02/39993, filed December 12, 2002, entitled ASSAYS AND IMPLEMENTS FOR DETERMINING AND MODULATING HSP90 BINDING ACTIVITY, which claims priority to United States Provisional Patent Application Ser. No. 60/340,762, filed December 12, 2001, and entitled ASSAYS FOR DETERMINING HSP90 BINDING ACTIVITY, each of which applications is herein incorporated by reference in its entirety.

Page 4, lines 1-5.

Recently, Nicchitta et al., WO 01/72779 (PCT/US01/09512), ~~demonstrated disclosed~~ that HSP90-GRP94 (an endoplasmic reticulum paralog of the Hsp90 family of chaperones) can assume a different conformation upon heat shock and/or binding by the fluorophore bis-ANS. Specifically, Nicchitta et al. demonstrated indicated that this induced conformation exhibits a higher affinity for certain HSP90 ligand stimulated polypeptide binding and chaperone activities than for relative to those exhibited by a different form of HSP90-GRP94 that predominates in normal untreated GRP94 cells.

Page 7, lines 26-28.

Figure 4 shows that 17-AAG (CF7) has a higher apparent binding affinity for HSP90 from tumor cells (BT474) than for HSP90 from normal cells (fibroblasts, RPTEC) ~~or and for purified native HSP90-alone~~, as determined using methods described herein.

Page 7, lines 29-30.

Figure 5 shows that 17-AAG (CF7) has a higher apparent binding affinity for HSP90s from the specific high Her-2 expressing cells, SKOV-3, SKBR-3, and N87, and for heat-shocked HSP90 and bis-ANS treated HSP90, than ~~from~~ for HSP90 from normal cells, ~~heat-shocked HSP90, or bis-ANS treated HSP90.~~

Page 8, lines 1-3.

Figure 6 shows the results binding affinity (as indicated by the IC<sub>50</sub>) of various test compounds to HSP90 in MCF7 cell lysate and to purified native HSP90, and the ratio of the binding affinities of the purified native HSP90 relative to that of the HSP90 in MCF7 cell lysate for each compound. The IC<sub>50</sub> reported is the concentration of the compound needed to cause half-maximal inhibition of binding of 17-AAG. ~~used in certain assays embodiments of the invention. The cell line used was MCF7.~~ Synthesis and use of the ~~modulators~~ test compounds shown ~~is~~ are described in U.S. Patent Application Ser. No. 60/367,055 and/or in International Application Ser. No. PCT/US02/29715.

Page 31, line 27 to page 32, line 13.

Purified native HSP90 protein (Stressgen) or cell lysates prepared in lysis buffer (20 mM Hepes, pH 7.3, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 100 mM KCl) were incubated in the absence or presence of CF7 (17-AAG) or test compound for 15 min at 4 °C. Biotinylated-geldanamycin (biotin-GM) was then added to the mixture as discussed previously, and the reaction was further incubated by rotating for 1 hr at 4 °C. BioMag™ streptavidin magnetic beads were then added to the mixture, and the reaction was incubated by rotating for another 1 hr at 4 °C. Tubes were placed on a magnetic rack, and the unbound supernatant removed. The magnetic beads were washed three times in lysis buffer, and the washes discarded. SDS-PAGE sample buffer was added to the beads and boiled for 5 min at 95°C. Samples were analyzed on 10% SDS protein gels (Novex), and then Western blots using anti-HSP90 monoclonal antibody (Stressgen SPA-830). The bands in the Western Blots were quantitated using the Bio-rad Fluor-S Imager, and

the % inhibition of binding of CF7 or test compound calculated. The IC<sub>50</sub> reported is the concentration of the compound needed to cause half-maximal inhibition of binding. For experiments that utilized heat-shocked HSP90, the purified HSP90 native protein was incubated for 15 min at 90°C. For experiments that utilized bis-ANS treated HSP90, the purified HSP90 protein was incubated with bis-ANS (Molecular Probes) for 30 min at 37 °C. The results are shown in Figures 4-6.

Page 33, lines 1-33.

Briefly, drug was administered in a single dose of 30 mg/kg body weight via the intraperitoneal route in a formulation comprising 6 parts PEG400, 1 part ethanol and 3 parts Tween 80 ("PET formulation"). Test animals (3/group) were sacrificed at the indicated times after dosing (as indicated in Figures 7 and 8). Tumors were excised, minced into 1 mm<sup>3</sup> fragments and frozen at -80 °C in cryovials until ready for use, at which time they were thawed and homogenized in acetonitrile, and the solvent then evaporated off under nitrogen. Extractions were then performed on the homogenate using ethyl acetate/water. Blood samples were taken at the times indicated in Figure 8 by serial bleed from the retro-orbital sinus or the tail vein. Blood was collected into tubes, allowed to clot at room temperature for one hour and serum was collected as the supernatant after centrifugation at 300 x g for 5 minutes. The usual volume of serum available for analysis from the pharmacokinetics studies is 15 µL, although smaller volumes can be used. 485 µL acetonitrile and 15 µL serum sample (from each time point) are added to a 1.5 mL siliconized microcentrifuge tube and the mixture vortexed briefly at high speed. The tubes are then centrifuged at 21,000 × g for 10 minutes, after which the organic layer is transferred to a glass test tube. The tubes containing the organic phase are then placed in a Turbovap set at 40 °C for 5-10 min. and the acetonitrile blown off using 25 psi nitrogen. Once the tubes are totally dry, the sample is reconstituted with 150 µL of mobile phase (30% Mobile Phase B: Acetonitrile w/ 1% Acetic Acid and 0.5% TEA/70% Mobile Phase A: H<sub>2</sub>O w/ 1% Acetic Acid and 0.5% TEA for ansamycins , 5% Mobile Phase B: Acetonitrile with 0.05% TFA/95% Mobile Phase BA: H<sub>2</sub>O with 0.1% TFA). The tubes are well mixed and the contents then

transferred to HPLC vials or 96-well microplates with a micro-insert. HPLC is then performed on the samples at 160 bar using an Agilent 1100 series device with a Zorbax 300SB-C<sub>18</sub> column (5 µM particle size; 150 x 4.6 mm), UV photodiode array detector, and the following phases of solvent (30% Mobile Phase B: Acetonitrile w/ 1% Acetic Acid and 0.5% TEA/70% Mobile Phase A: H<sub>2</sub>O w/ 1% Acetic Acid and 0.5% TEA for ansamycins, 5% Mobile Phase B: Acetonitrile with 0.05% TFA/95% Mobile Phase A: H<sub>2</sub>O with 0.1% TFA). The analysis takes approximately 15-25 minutes per sample, based on method. As controls, spiked serum standards using pooled Balb/C or Nu/Nu mouse serum containing 10, 25, 50, 500 and 5000 ng/mL of the compounds of interest were run for each analytical run per the following table.

Page 36, lines1-3

| Time<br>Hour | <del>CF-237</del><br><del>BT 474</del><br>mean | CF-237<br>RPTEC | CF-237<br>HUVEC | CF-237 BT<br>474-01 | CF-237 BT<br>474-02 | <del>CF-237</del><br><del>BT 474</del><br>mean | <del>CF-237</del><br><del>BT 474</del><br>SD |
|--------------|--|-----------------|-----------------|---------------------|---------------------|--|--|
| 0.5          | 8570   | 203             | 2603            | 8838                | 8302                | 8570   | 379.07                                       |
| 2            | 26500  | 894             | 7470            | 26759               | 26242               | 26500  | 365.37                                       |
| 4            | 41647  | 905             | 9100            | 42530               | 40764               | 41647  | 1248.61                                      |
| 8            | 61970  | 1520            | 15483           | 57883               | 66056               | 61970  | 5779.42                                      |
| 16           | 101902   | 1450            | 16706           | 77287               | 126517              | 101902   | 34810.46                                     |
| 24           | 97677  | 1485            | 11026           | 94726               | 100628              | 97677  | 4173.41                                      |